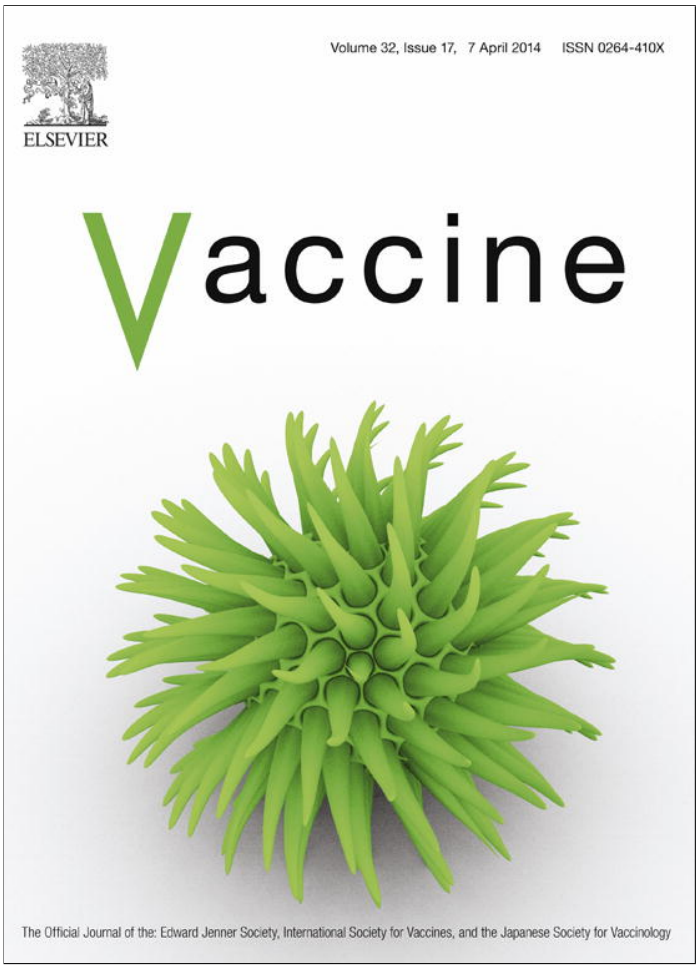

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The effects of administration of ligands for Toll-like receptor 4 and 21 against Marek's disease in chickens

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ABSTRACT

Ligands for Toll-like receptors (TLRs) are known to stimulate immune responses, leading to protection against bacterial and viral pathogens. Here, we aimed to examine the effects of various TLR ligands on the development of Marek's disease in chickens. Specific-pathogen free chickens were treated with a series of TLR ligands that interact with TLR3, TLR9 and TLR21. In a pilot study, it was determined that TLR4 and TLR21 ligands are efficacious, in that they could reduce the incidence of Marek's disease tumors in infected birds. Hence, in a subsequent study, chickens were treated with lipopolysaccharide (LPS) as a TLR4 and CpG oligodeoxynucleotides (ODN) as TLR21 agonists before being challenged with the RB1B strain of Marek's disease virus (MDV) via the respiratory route. The results demonstrated that the administration of LPS or CpG ODN, but not PBS or non-CpG ODN, delayed disease onset and reduced MDV genome copy number in the spleens of infected chickens. Taken together, our data demonstrate that TLR4 and 21 agonists modulate anti-virus innate immunity including cytokine responses in MD-infected chicken and this response can only delay, but not inhibit, disease progression.

1. Introduction

Marek's disease (MD) is caused by an alphaherpesviurs named Marek's disease virus (MDV) [28]. MDV enters the body through the respiratory tract and it is transported to lymphoid organs by macrophages and B cells [6,7]. The early cytolytic phase results in B cell death, which occurs around 2–7 days post-infection (d.p.i.) [11]. MDV then infects activated T cells and enters its latency phase at around 7–10 d.p.i. The time points in the present study were selected based on the above model to represent different phases of MDV pathogenesis.

Immunity against MDV is mediated by innate and adaptive immune mechanisms. Among innate defense mechanisms, Toll-like receptors (TLRs) play an important role in recognition of the virus and elicitation of innate and adaptive immune responses against the virus [2]. TLRs are pattern recognition receptors (PRR) that recognize conserved pathogen-associated molecular patterns (PAMPs) [21] and trigger pro-inflammatory cytokine production such as interleukin (IL)-1β and IL-6 and type I interferons [44].

A repertoire of TLRs orthologous to those of mammalian TLRs has been described in chickens [24,32]. The avian orthologue of TLR4 is required for the recognition of LPS [22], up-regulation of interferon (IFN)-γ [35,42] and increased expression of pro-inflammatory cytokines in chickens [39,45]. TLR4 agonists can be used prophylactically to elicit immunity against pathogens such as influenza viruses in chickens or mice [27,41].

Bacterial and viral DNA has unmethylated CpG motifs that are recognized by TLR9 in mice and humans [44], and by TLR21 in chickens [10]. CpG ODN induces the expression of IL-1β and IFN-γ in avian macrophages [18] and stimulates the proliferation of chicken B cells in vitro [47]. The induction of T helper (Th)1 immune responses, characterized by expression of IFN-γ, has been demonstrated in neonatal chickens treated with CpG-ODN [31,40]. CpG ODN has been used prophylactically to induce protective immunity against *Escherichia coli* [16], *Salmonella* [43], and avian influenza infections in chickens [42].

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Considering the ability of TLR agonists to induce immune responses in chickens, the present study was conducted to test the hypothesis that treating chickens with TLR3, 4 or 21 agonists can confer immunity against MDV.

2. Materials and methods

2.1. Experimental animals

Specific-pathogen free chicks were obtained from the Canadian Food Inspection Agency (Ottawa) and kept in Horsfal units placed at the Isolation Unit of Ontario Veterinary College (University of Guelph, Guelph, Ontario, Canada). Animals were euthanized by CO_2 inhalation and procedures were approved by the institutional Animal Care Committee.

2.2. TLR ligands

Lipopolysaccharide (LPS) from *E. coli* 0111:B4 and poly(I:C) were purchased from Sigma-Aldrich Canada (Oakville, Canada). The synthetic class B CpG ODN 2007 [5-CGTCGTTGTCGTTTTGTCGTT-3] and non-CpG ODN [5-TGCTGCTTGTGCTTTTGTGCTT-3] [10] were purchased from Eurofins MWG Operon (Ebersberg, Germany). FimH was kindly provided by Dr. Ali Ashkar (McMaster University, Hamilton, Canada). All of the ligands used were re-suspended in sterile PBS, pH 7.4.

2.3. MDV infection

2.3.1. Virus strain

The very virulent (vv) MDV strain, RB1B (passage 9) was used to infect the birds via inhalation (provided by Dr. K.A. Schat, Cornell University, NY, USA). Extraction and administration of cell-free MDV was done following the protocol described previously [12] with some modification as described elsewhere [3].

2.3.2. Experimental design

Two independent trials were performed using 210 day-old chickens in total. In the first trial, 4 day-old chickens ($n = 100$) were treated with LPS (High dose: 500 μg/bird and low dose 100 μg/bird), CpG ODN 2007 (High dose: 10 μg/bird and low dose: 2 μg/bird), non-CpG ODN (10 μg/bird), FimH (high dose: 50 μg/bird and low dose: 10 μg/bird), and poly(I:C) (400 μg/bird) via the intra-air sac route (i.a.s.). The birds were challenged 24 h after TLR administration by inhalation of cell-free RB1B strain of MDV (using a virus preparation containing 1280 pfu of MDV/ml). A group of age-matched chickens were treated with un-infected skin extract plus PBS (sham infected group). On 14 d.p.i., the chickens were treated intra-muscularly (I.M.) with the same doses of TLR ligands. In the second trial, 4 day-old birds ($n = 110$) were treated with LPS (500 μg/bird), CpG ODN 2007 (10 μg/bird), non-CpG ODN (10 μg/bird)) or diluent (PBS) via the i.a.s. route. The birds were infected with RB1B 24 h after the treatment, as described above. Similar to the trial 1, the birds were treated I.M with the same dose of TLR ligand on day 14 post infection.

The chickens were monitored three times a day after infection and scored based on the clinical signs such as ruffled feathers, huddling, droopy wings and paralysis. Chickens showing each of the first three clinical signs would receive a score of 1. Chickens that suffered from paralysis for more than 48 h or had the cumulative score of 3 based on other clinical signs had to be euthanized, according to the University of Guelph Animal Care Committee regulations. In the first trial, all birds were euthanized on day 21 post-infection, regardless of the presence or absence of clinical signs. After euthanasia, birds were necropsied and presence of gross tumors and enlargement of the sciatic nerve were recorded.

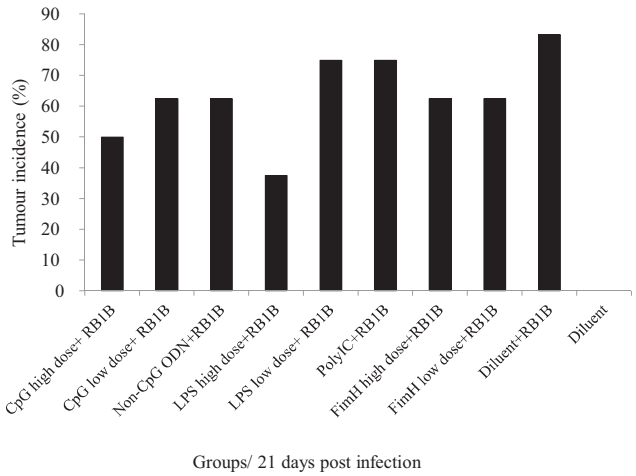

Fig. 1. The effects of TLR ligand treatments on tumor incidence in different groups at 21 d.p.i. (first trial). On day 4 post-hatch, chicks were treated with LPS (100–500 μg/bird), CpG ODN 2007 (2–10 μg/bird), non-CpG ODN (10 μg/bird), FimH (10–50 μg/bird), poly(I:C) (400 μg/bird) or diluent via the intra-air sac route (i.a.s.). Chickens were infected with RB1B via respiratory route on day 5 post-hatch. A group of birds received diluent but were not challenged with the virus. The treated birds were also received the same TLR ligand I.M. on day 14 post infection. The data are presented as the percentage of tumor incidence with eight biological replicates in each group at 21 d.p.i.

The chickens that had at least one gross visceral tumor or had an enlarged nerve were considered positive for MD. Feather tips and spleens were collected on 4, 10, and 21 d.p.i. In the second trial, 6 birds from each group were euthanized on day 4 and 10 d.p.i. and the rest of the birds were euthanized upon manifestation of MD signs (being on days 21, 27 and 28 d.p.i. in different groups) based on the above scoring system.

2.3.3. Real-time RT-PCR

RNA extraction from spleen and lung samples, cDNA synthesis and real-time RT-PCR were conducted as previously described [1]. Real-time RT-PCR was used to quantify the expression levels of IFN-α, IFN-β, IFN-γ, IL-1β, IL-18 and β-actin using the LC480 instrument (Roche Diagnostics GmbH, Mannheim, Germany) [4,9,46]. MDV genome copy number was quantified by measuring MDV meq gene copies in 100 ng of DNA extracted from tissues as described previously [5]. Cycling parameters were as previously described [9,46].

2.4. Data analysis

LightCycler® 480 relative quantification software was used for calculating relative expression. The expression of each cytokine was described relative to β-actin gene expression, which was used as the reference gene, in the same sample preparation. Data were analyzed using ANOVA and comparisons were considered significant at $p \leq 0.05$.

3. Results

3.1. The effects of TLR ligands on mortality and tumor incidence of chickens infected with MDV RB1B

The first trial was conducted to screen a set of different TLR agonists (CpG ODN, LPS, PolyIC and FimH) for their ability to inhibit tumor incidence induced by MDV. In this trial, 83.3% of RB1B inoculated-birds developed tumors on day 21 post-infection (Diluent/RB1B group), while no tumor incidence was observed in the control group (non-infected birds) (Fig. 1). The lowest

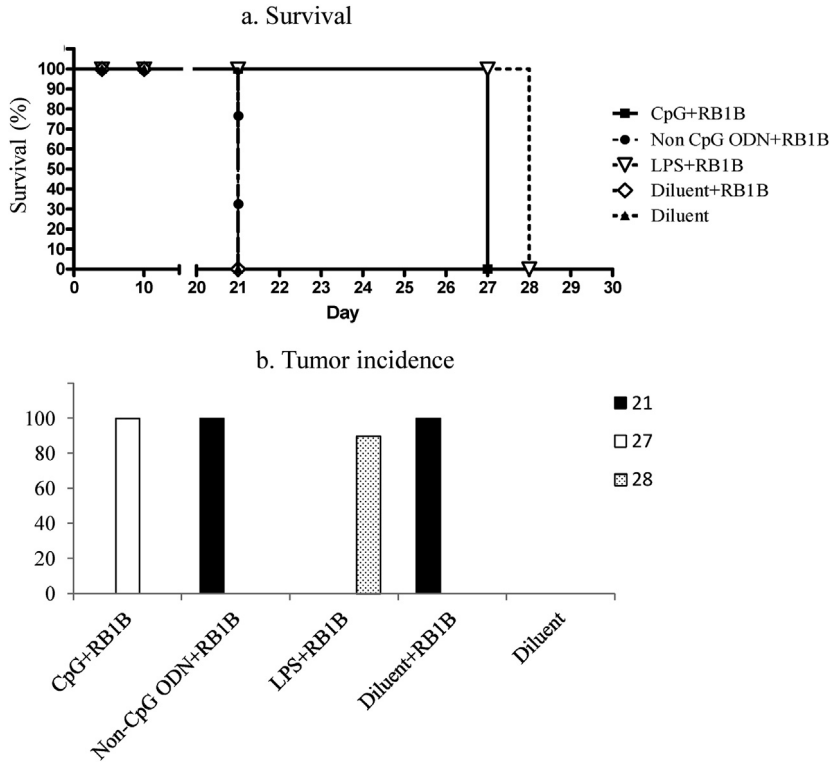

Fig. 2. The effects of TLR ligand treatments on survival rate and tumor incidence (second trial). Birds were treated with LPS, non-CpG ODN, CpG ODN or the diluent on day 5 post-hatch and were challenged with RB1B via respiratory system 24 h later. Birds were euthanized based on the presence of clinical signs, especially paralysis, and the survival rate for each group was calculated (a). The percentages of tumor incidence in different experimental groups are presented for day 21, 27, and 28 post infections. The data are presented as the percentage of tumor incidence in ten biological replicates in each experimental group (b).

tumor incidence (37.5%) on day 21 post-infection was noted in the chickens which were administered with the high dose of LPS (500 μg/bird) (LPS high dose/RB1B group), demonstrating that LPS administration can reduce tumor incidence. The administration of Poly(I:C), FimH, non-CpG ODN or CpG ODN only marginally reduced tumor incidence compared to the non-treated group (Diluent/RB1B) (Fig. 1).

Based on the results of the first trial, certain ligands and doses were selected. In the second trial, the birds received CpG-ODN, non-CpG-ODN, LPS or PBS (diluent) prior to infection with RB1B and birds were monitored for survival rate and tumor incidence. All the birds receiving non-CpG-ODN or the diluent (non-CpG/RB1B or diluent/RB1B groups) showed clinical signs of MD by day 21 post-infection and upon necropsy, they all had gross visceral tumors. In contrast, on day 21 post-infection, no MD clinical signs were observed in the birds administered with LPS or CpG ODN. In CpG/RB1B and LPS/RB1B groups, the birds remained free of clinical signs until day 25 post-infection at which time the majority of the chickens in these groups began to show clinical signs of MD, reaching the cumulative score of 3 at 27 and 28 d.p.i., respectively. Therefore, they had to be euthanized. Upon necropsy, all the birds were found to have gross visceral lesions consistent with MD tumors (Fig. 2a and b).

3.2. Relative gene expression of cytokines and absolute quantification of MDV genome load in the lungs

In the second trial, we analysed the expression of IFN-α, IFN-β, IFN-γ, IL-1β and IL-18 in the lungs of birds from different experimental groups (Fig. 3a–e). The results demonstrated that there was no difference in the expression of IFN-α and IL-18 among the groups at 4 and 10 d.p.i. IFN-β expression was up-regulated in the LPS/RB1B and non-CpG/RB1B groups compared to the other groups at 4 d.p.i. ($p = 0.021$). IFN-γ expression was significantly up-regulated in all groups at 4 and 10 d.p.i. compared to the diluent group ($p = 0.015$). IL-1β expression was up-regulated in the CpG/RB1B and LPS/RB1B groups at 4 d.p.i. compared to the other groups ($p = 0.016$). MDV genome load in CpG/RB1B ($p = 0.002$) and LPS/RB1B ($p = 0.05$) groups was higher on day 10 post-infection than that on day 4 post-infection. However, there was no difference in MDV genome load among the experimental groups in the same time point (Fig. 3f), suggesting that LPS or CpG ODN treatments did not alter viral load in the lungs.

3.3. Expression of cytokines and quantification of MDV genome load in spleen (second trial)

In the second trial, cytokine expression was analyzed in spleens of birds from different experimental groups (Fig. 4a–e). There was no statistical difference among the groups in the expression of IFN-α, IFN-β and IL-18 at 4 and 10 d.p.i. The expression of IL-1β in CpG/RB1B, non-CpG/RB1B and LPS/RB1B groups was significantly higher at 4 d.p.i. compared to other groups ($p = 0.02$). MDV genome load was significantly higher at 4 d.p.i. in non-CpG/RB1B and diluent/RB1B groups compared to other groups ($p = 0.005$) (Fig. 4f), demonstrating that the administration of CpG or LPS reduces MDV viral load in the spleen.

4. Discussion

TLR ligands have been employed as vaccine adjuvants or as stand-alone prophylactic anti-viral compounds for control of viral or bacterial infections. For example, LPS and FimH have been used prophylactically against influenza virus in the mouse model and have been shown to control morbidity and mortality caused by this virus [1,38]. In chickens, LPS, CpG and poly(I:C) have been tested

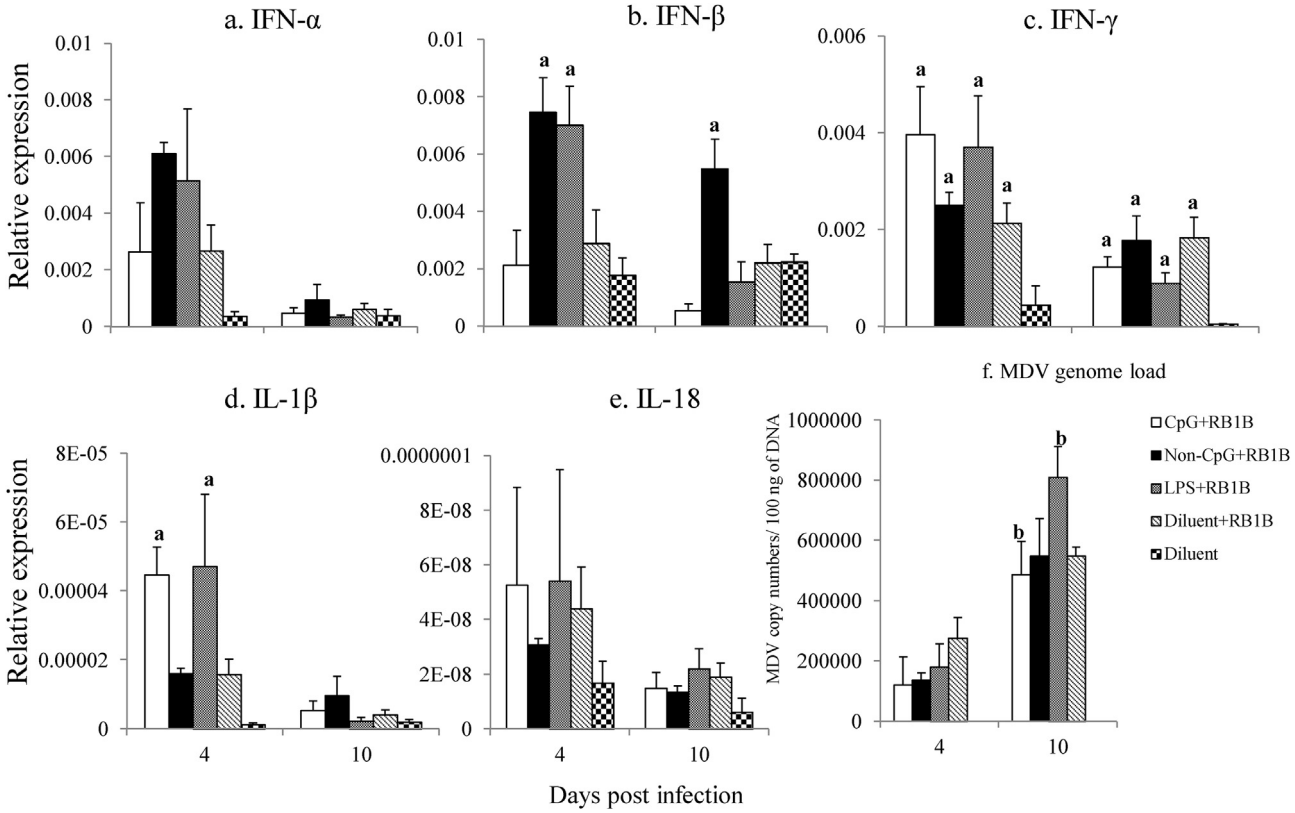

Fig. 3. Relative expression of IFN-α, IFN-β, IFN-γ, IL-1β, IL-18 to β-actin in the lungs of birds from the second trial. The expression of the cytokines was calculated relative to β-actin in the lungs of birds treated with LPS (500 μg/bird), CpG ODN (10 μg/bird), non-CpG ODN (10 μg/bird) or the diluent and were challenged with RB1B via respiratory route. The data show the mean ± standard error of six biological replicates for IFN-α (a), IFN-β (b), IFN-γ (c), IL-1β (d), IL-18 (e) at 4 and 10 d.p.i. (f) demonstrates MDV genome copy number (using MDV meq gene) in the lungs. The data show the mean ± standard error of six biological replicates for each group at 4 and 10 d.p.i. "a" Denotes statistical significance compared to other groups at the same time point. "b" Denotes statistical significance for one group compared to other time points for the same group. Comparisons were considered significant at $p \leq 0.05$.

for their efficacy as prophylactic compounds against bacterial or viral pathogens. CpG-ODN can protect chickens against bacterial pathogens such as *E. coli* [16] as well as viral pathogens such as infectious bronchitis virus [13]. Furthermore, our group evaluated the efficacy CpG-ODN, poly(I:C) and LPS against avian influenza virus (AIV) infection in chickens and concluded that poly(I:C) was the most effective TLR agonist for controlling AIV replication [42]. The present study investigated the efficacy of a series of TLR agonists, including poly(I:C), FimH, LPS and CpG, to raise immunity against MDV.

There were two trials in this study. In the first trial, we screened different TLR ligands and doses to identify the optimal ligand and the dose that could confer immunity against MDV. The doses used in the first trial were chosen based on the results obtained from previous experiments with these TLR ligands [1,16,27,30,40]. We used a protocol for ligand treatments, which consisted of two administrations, first via the intra-air sac route and the second via the intramuscular route. The Intra-air sac route was chosen because we reasoned that elicitation of local innate immune response in the air sacs can delay the entrance of the viral particles into the lungs. Importantly, in avian species, the inhaled air passes through air sacs before entering the lungs [33], providing a direct contact between air sacs and MDV. The I.M. route was used for administering the second dose (day 14 post-infection) because we reasoned that this time point corresponds to the end of the latency phase and its reactivation. We reasoned that this may be a relevant time for induction of systemic host innate responses to disrupt the virus cycle and block the transformation phase.

The second trial was designed to replicate the results of the first trial and also to provide further insights into the mechanisms of protection. The results of the first trial revealed that treatment of chickens with high dose of LPS or CpG could lead to lower incidence of tumors by 21 d.p.i. Other ligands also had some protective effects, albeit at varying levels. For example, poly(I:C) and FimH were not as effective as other ligands in reducing the tumor incidence, although poly(I:C) can enhance the efficacy of vaccines against Marek's disease [29]. The lack of efficacy in case of poly(I:C) and FimH could be due to the route of administration or their dose. It is also possible that these ligands were degraded before interacting with their receptors. Given the relatively potent effects of CpG and LPS at high doses, these two ligands were selected for the subsequent trial.

The results of the second trial further confirmed that treatment of chickens with CpG and LPS via i.a.s. and I.M. routes prior to infection with MDV delays disease onset in the treated chickens by 6 and 7 days, respectively, compared to the control groups.

To identify the immunological correlates of TLR ligand-mediated effector functions, the expression of several cytokines was measured in spleen and lungs. The expression of IFN-β, a type I interferon, was significantly up-regulated in LPS/RB1B group at 4 d.p.i. in the lungs. Triggering the TLR4-associated pathways elicits the expression of type I IFNs, especially IFN-β [44]. Mammalian type I IFNs have potent antiviral activity, increase the expression of TLRs [34], and induce T helper (Th)1 immune response [8]. In chickens, type I interferons also have anti-viral activities [36] and we have recently shown that type I IFN genes are up-regulated at early hours post-treatment with LPS in chickens [40]. Therefore, our observation of up-regulation of IFN-β at early time points in the lungs may

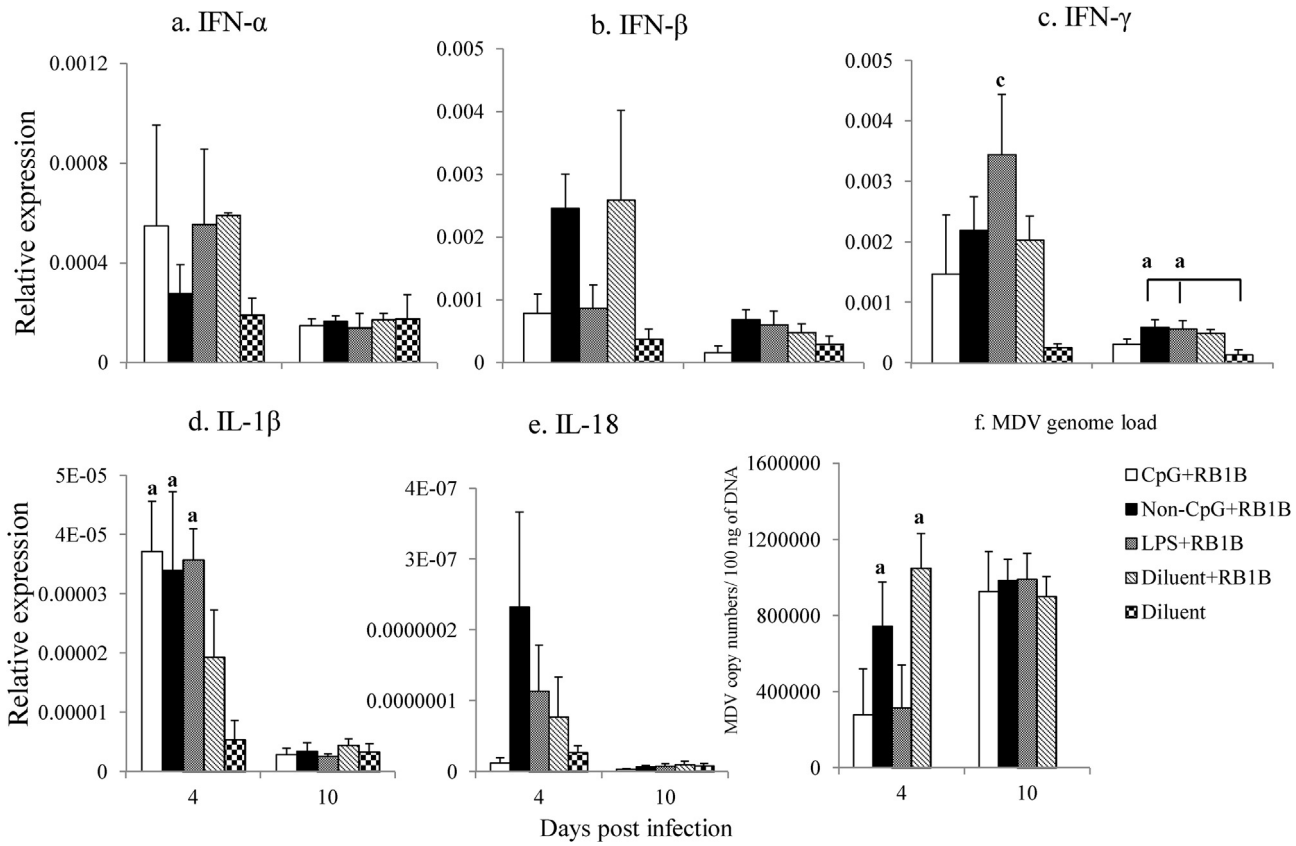

Fig. 4. Relative expressions of IFN-α (a), IFN-β (b), IFN-γ (c), IL-1β (d), IL-18 (e) in the spleen of birds from the second trial are shown. 4-day old birds were injected with LPS (500 μg/bird), CpG ODN (10 μg/bird), non-CpG ODN (10 μg/bird) or the diluent and were challenged with RB1B via respiratory route on day-5 post hatch, as described in detail in Section 2. On 14 d.p.i., chickens were treated with the same doses of TLR ligands intra-muscularly and the expression of cytokines was calculated relative to β-actin. Part (f) demonstrates MDV genome copy number in the spleens of the experimental groups. The data represent the mean of six biological replicates for each group at 4 and 10 d.p.i. "a" Denotes statistical significance compared to other groups at the same time point. "b" Denotes statistical significance for one group compared to other time points for the same group. Comparisons were considered significant $p \leq 0.05$.

be an important contributing factor to host response generated by LPS treatment. Furthermore, the up-regulation of type I IFN (at 4 d.p.i. in LPS/RB1B group) coincided with the early cytolytic phase of MDV in the lungs. Therefore, administration of LPS via the i.a.s. route might play a role in eliminating MDV viral particles. This is indicated by the low MDV genome copy number at 4 d.p.i., in the lungs, which might have contributed to the delay in the manifestation of clinical signs and pathologic lesions of MD. There was, however, no difference in type I IFN expression in spleen among various groups. Our finding is supported by previous reports that local delivery of TLR ligands does not result in measurable production of type I IFN [14]. Moreover, absence of IFN-β secretion has been reported in vaccinia virus infection, where TLR ligands were used to confer protection [20].

Administration of CpG via the i.a.s. route had no effect on the transcription of type I IFNs at 4 d.p.i. in the lungs or spleens. This might have occurred due to several reasons. First, the expression of TLR21 is very low in the lungs compared to other tissues [10]. Second, TLR21, similar to its mammalian counterpart TLR9, is an intracellular receptor [10] which might have made it inaccessible for at least some of the administered CpG. And thirdly, the half-life of CpG in vivo is short [26] due to its rapid degradation. Finally, class B CpG that was used in our experiments is a poor inducer of type I IFN [25]. On the other hand, non-CpG induced the expression of type I IFNs and IFN-γ in the lungs. Non-CpG also induced the expression of inflammatory cytokines in spleen. The immune stimulatory effects of non-CpG have been noted both in B cells of mammals and chicken [37,47]. However, the induction of IFNs and pro-inflammatory cytokines by non-CpG in our experiments had no effect on MDV genome load and onset of clinical disease in this group. It is of note that in the context of influenza virus, we have shown that non-CpG may even have some protective effects [41].

In addition to type I IFNs, IFN-γ and pro-inflammatory cytokines can play a pivotal role in elicitation of protective immune responses against MDV [15,17]. IFN-γ could block MDV replication via activation of immune system cells and production of NO [48] and enhancement of cytotoxic T cell (CTL) responses [17]. Based on our results, administration of LPS to birds resulted in a significant increase in the expression of IFN-γ in the lungs at 4 and 10 d.p.i. and in spleen at 10 d.p.i. Administration of CpG to chickens also led to a significant increase in IFN-γ expression in the lungs at 4 and 10 d.p.i. Based on previous observations, CpG induces the expression of IFN-γ [23] and elicits Th1immune responses in the mouse model [49]. Taken together, induction of IFN-γ expression by LPS and CpG in the lungs and spleen may play a role in the development of protective immune responses against MDV.

Our results demonstrated that the administration of LPS and CpG resulted in a significant increase in the expression of pro-inflammatory cytokines, represented by IL-1β in both the lungs and spleens of MDV-infected chickens. This is in agreement with the results demonstrating that LPS and CpG stimulate the up-regulation of pro-inflammatory cytokines in chicken cells in vitro [19]. IL-1β is one of the early innate genes that is up-regulated in response to MDV infection. In fact, up-regulation of IL-1β has been noted in the spleen and lungs of chickens infected with MDV [2,48]. There

is, however, little known about the importance of this cytokine in immunity against MD.

In conclusion, administration of TLR agonists such as LPS and CpG enhances immunity against MD. Despite enhancement of immunity, we determined that treatment with TLR agonists did not reduce virus burden in feathers. This requires further investigation and perhaps better formulations of TLR agonists and delivery systems need to be devised to induce immunity against disease and also reduce virus load, especially in the feather.

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